Experimental evidence for the ectodermal origin of the epithelial anlage of the chicken bursa of Fabricius

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SUMMARY
The bursa of Fabricius (BF) is a central lymphoid organ of birds responsible for B-cell maturation within bursal follicles of epithelial origin. Despite the fundamental importance of the BF to the birth of B lymphocytes in the immune system, the embryological origin of the epithelial component of the BF remains unknown. The BF arises in the tail bud, caudal to the cloaca and in close association with the cloacal membrane, where the anal invagination (anal sinus) of ectoderm and the caudal endodermal wall of the cloaca are juxtaposed. Serial semi-thin sections of the tail bud show that the anal sinus gradually transforms into the bursal duct and proctodeum, which joins the distal part of the cloaca during late embryogenesis. These anatomical findings raise the possibility that the ectoderm may contribute to the epithelial anlage of the BF. The expression of sonic hedgehog and its receptor in the embryonic gut, but not in the BF, further supports an ectodermal origin for the bursal rudiment. Using chick-quail chimeras, quail tail bud ectoderm was homotopically transplanted into ectoderm-ablated chick, resulting in quail-derived bursal follicle formation. Chimeric bursal anlagen were generated in vitro by recombining chick bursal mesenchyme with quail ectoderm or endoderm and grafting the recombination into the chick coelomic cavity. After hematopoietic cell colonization, bursal follicles formed only in grafts containing BF mesenchyme and tail bud ectoderm. These results strongly support the central role of the ectoderm in the development of the bursal epithelium and hence in the maturation of B lymphocytes.

KEY WORDS: Bursa of Fabricius, Ectoderm, Chicken, Chimera, Tail bud

INTRODUCTION
The secondary lymphoid organs develop from the mesoderm, but they communicate with the environment via ectoderm and endoderm. The primary lymphoid organs, such as the thymus and bursa of Fabricius (BF) of birds, develop in genetically determined locations, where the ectoderm and endoderm are juxtaposed at the branchial pouches and cloacal membrane, respectively. The functional framework for both primary lymphoid organs is the lympho-epithelial (LE) tissue, in which the stellate-shaped epithelial cells form a 3D meshwork. This structure creates a micro-environment for T- and B-cell maturation (Glick, 1985; Glick and Oláh, 1993; Cooper et al., 2006). The epithelial anlage of the thymic primordium is of endodermal origin (Jolly, 1915; Hammond, 1954; Le Douarin and Jotereau, 1975; Gordon et al., 2004) and completely separated from the body surface. By contrast, the BF is connected via the bursal duct to the third portion of the cloaca: the proctodeum (Boyden, 1922; Romanoff, 1960; Oláh et al., 1986).

In mammals, the cloaca partitions into a ventral urogenital sinus and a dorsal anal canal. In birds, the cranio-caudal differentiation of the cloaca results in cranial coprodeum (continuation of the hindgut endoderm) and caudal urodeum, where the ureters enter. The third part of the cloaca is the proctodeum, which receives the bursal duct and develops from the ectodermal anal invagination. The proctodeum joins the urodeum in late embryogenesis: at E17. These anatomical connections raise the possibility of an ectodermal origin for the bursal epithelial primordium (Retterer, 1885; Minot, 1900; Oláh et al., 1986), but experimental evidence for this has been lacking.

In adult birds, the BF, a chestnut-sized organ between the cloaca and the sacrum, is the site of B-lymphocyte differentiation (Glick, 1985; Glick, 1991). Bursal differentiation can be staged as follows: (1) appearance of the pre-programmed epithelial anlage in the tail bud mesenchyme around E4.5-E5; (2) follicle formation, when hematopoietic cells enter the bursal mesenchyme and subsequently the surface epithelium, establishing LE tissue between E11 and E14; (3) follicle-associated epithelium differentiation at E15; and (4) development of cortical region after hatching. This study focuses on the first two stages of bursal development. These are crucial stages because the functional activity of the BF, namely B-cell differentiation, requires formation of bursal follicles and LE tissue. The bursal epithelial primordium is generally believed to be a cloacal diverticulum of endodermal origin (Wenkebach, 1888; Jolly, 1915; Boyden, 1922; Pera, 1958; Miller and Brigin, 1996). The vacuolization of the cloacal plate endoderm has been postulated to give rise to the epithelial rudiment (Boyden, 1922; Miller and Brigin, 1996). However, the pluripotent mesenchymal cell mass (rest of Hensen’s node) of the tail bud is confluent with the overlying ectodermal and underlying endodermal cells (Schoenwolf, 1979; Schoenwolf, 1981), which makes the origin of the bursal epithelial primordium uncertain.

The purpose of this study was to determine the origin of the bursal epithelial anlage. Using chick-quail chimeras and tissue recombination, we provide experimental evidence for the ectodermal origin of the bursal epithelial primordium. This finding contributes to our understanding of the micro-environment that influences B lymphocyte differentiation.

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MATERIALS AND METHODS

Animals
Fertilized White Leghorn chicken and quail (Coturnix coturnix japonica) eggs were obtained from commercial breeders and maintained at 37°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH) tables (Hamburger and Hamilton, 1992) or the number of embryonic days (E). The design and condition of the animal experiments were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary.

Histological procedures
The tissue samples were fixed in 4% buffered glutaraldehyde and embedded in Polybed/Araldite 6500 mixture (Polyscience, Warrington, PA). The 1 μm semi-thin sections were stained by Toluidine Blue.

Immunocytochemistry
Avian embryos and the coelomic grafts were fixed in 4% paraformaldehyde, embedded in gelatin and 10 μm frozen sections placed onto poly-L-lysine coated slides (Sigma-Aldrich, Hungary). The primary antibodies are listed in Table 1.

Sections were incubated with primary antibodies for 45 minutes, followed by biotinylated goat anti-mouse IgG (Vector Labs, Burlingame, CA) and avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Labs). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (Sigma) for 10 minutes. The binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol (Sigma).

For double-immunofluorescence, sections were incubated with primary and fluorescent-labeled secondary antibodies: anti-mouse IgG Alexa Fluor 594, anti-mouse IgG2a Alexa Fluor 488; anti-mouse IgG1 Alexa Fluor 594 (Molecular Probes); and anti-mouse IgG Pacific Blue (Jackson ImmunoResearch). Images were compiled using Adobe Photoshop.

In situ hybridization
In situ hybridization was performed for chick sonic hedgehog (Shh) and Pax1 on 12 μm frozen sections using digoxigenin-labeled riboprobes [plasmids provided by Cliff Tabin (Riddle et al., 1993; Roberts et al., 1998)]. Riboprobe synthesis and in situ hybridization were performed as previously described (Nagy and Goldstein, 2006).

Ablation of tail bud ectoderm
The tail bud ectoderm of 15-16 HH stage (E2.5) chick embryos was labeled with 2% Nile Blue Sulphate in PBS and the epithelium removed with a tungsten needle. Control embryos received only Nile Blue Sulphate solution. Embryos were collected at 8 days after surgery. Bursal epithelial primordium was labeled with anti-cytokeratin staining and hematopoietic cell colonization was marked with CD45 monoclonal antibody.

Chick-quail tail bud chimera
To clarify the contribution of ectoderm to BF, quail tail bud ectoderm was grafted in ovo homotopically to chick embryo after tail bud ectoderm ablation. The operation was performed on HH stages 14-15 embryos, caudal to the 24-25th somites. The quail (donor) embryo was isolated from the yolk and the tail bud ectoderm with the associated mesoderm removed with tungsten needle. Microsurgery of the chick embryo (host) was performed in ovo. After windowing the eggshell and the soft shell membrane, a slit was made on the vitelline membrane covering the prospective tail bud region. The tail bud ectoderm, neural tube and the associated somatopleural mesoderm were discarded and the quail tail bud ectoderm, with the neural tube and mesoderm, was transferred to the chick embryo (see Fig. S1 in the supplementary material). After careful orientation of the graft, the chimeric embryos were incubated for a further 12 days.

In vitro recombination of chicken bursal mesenchyme with quail ectoderm and endoderm
Chick BF is colonized by hematopoietic cells at E9-E10. We therefore selected E8 bursal mesenchyme for tissue recombination. Tail bud ectoderm and endoderm from 15 HH stage, trunk ectoderm from 10 HH stage, hindgut endoderm from 27 HH stage and E8 bursa epithelium of quail embryos were recombined with E8 chick bursal mesenchyme. For separation of epithelium from mesenchyme, tissues were incubated in DMEM containing 0.03% collagenase (Sigma) at 37°C for 15 minutes. After enzymatic digestion, tissues were extensively washed with DMEM containing 10% fetal calf serum (Gibco), 5% chicken serum (Sigma), glutamine, non-essential amino acids, 100 U/ml penicillin G and 0.1 mg/ml streptomycin mixture to inactivate the collagenase activity. The in vitro tissue recombintants were embedded into a three-dimensional collagen gel matrix (BD Bioscience) described previously (Nagy and Goldstein, 2006). After overnight incubation, the recombintant chimeric epithelial-mesenchymal anlage was removed from the collagen gel and immunocytochemically checked for chicken and quail cells by 8F3 and QCPN mAb, respectively. The recombintant tissue was implanted into E3 (HH19 stage) chick coelomic cavity (Nagy et al., 2004). During this incubation, chick hematopoietic cells colonized the implanted ‘artificially’ produced bursal epithelial-mesenchymal primordium. After 14 days of incubation, the grafts were removed and checked for bursal follicle and LE formation. These studies included a total of 69 chimeric experiments comprising three separate series.

Table 1. Primary antibodies

<table>
<thead>
<tr>
<th>Cells/structures identified</th>
<th>Antibody specificity</th>
<th>Species specificity</th>
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<th>Source</th>
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<tr>
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<td>Unknown nuclear antigen</td>
<td>Quail</td>
<td>Clone QCPN (mouse IgG1)</td>
<td>DSHB</td>
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<tr>
<td>Chicken cells</td>
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<td>Clone 8F3 (mouse IgG1)</td>
<td>DSHB</td>
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<tr>
<td>Hematopoietic and endodermal cells</td>
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<td>Quail</td>
<td>Clone QH1 (mouse IgG1)</td>
<td>DSHB</td>
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<td>Hematopoietic cells</td>
<td>CD45</td>
<td>Chick</td>
<td>Clone HISC7 (mouse IgG2a)</td>
<td>Cedi Diagnostics, The Netherlands</td>
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<tr>
<td>Dendritic cells</td>
<td>Unknown antigen</td>
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<td>Clone 74.3 (mouse IgG1)</td>
<td>Cedi Diagnostics, The Netherlands</td>
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<tr>
<td>Antigen presenting cells</td>
<td>MHC II</td>
<td>Chick</td>
<td>Clone TAP1 (mouse)</td>
<td>DSHB</td>
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<tr>
<td>B lymphocytes</td>
<td>IgM</td>
<td>Chick</td>
<td>Clone M1 (mouse IgG2b)</td>
<td>Southern Biotech</td>
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<tr>
<td>B lymphocytes</td>
<td>Bu-1 antigen</td>
<td>Chick/quaill</td>
<td>Clone BoA1 (mouse IgG1)</td>
<td>Igyarto et al., 2008</td>
</tr>
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<td>B lymphocytes</td>
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<td>Quail</td>
<td>Clone L22 (mouse IgG1)</td>
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<td>Clone 11G2 (mouse IgG1)</td>
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<td>Clone 36 (mouse IgG2a)</td>
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<td>Chick/quaill</td>
<td>Clone Lu5 (mouse IgG1)</td>
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<td>Endoderm</td>
<td>Sonic hedgehog</td>
<td>Chick/quaill</td>
<td>Clone SE1 (mouse IgG1)</td>
<td>DSHB</td>
</tr>
</tbody>
</table>

DSHB, Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by University of Iowa, Dept of Biological Sciences, Iowa City, IA 52242, USA).
RESULTS AND DISCUSSION
Formation of the proctodeum, bursal duct and epithelial anlage of the BF
The epithelial rudiment of the BF emerges in the tail bud mesenchyme at around E4-E5 days. In the E5 chicken embryo, the thickened cloacal membrane (anal plate) is located between the anal sinus and cloaca (Fig. 1A). By E8, the anal sinus transforms into cranial and caudal portions from which the bursal duct and the proctodeum, respectively, develop (Fig. 1B). At this stage, the bursal epithelial anlage is a vesicle-like structure and the duct is still separated from the cloaca by the cloacal membrane (Fig. 1B). During the next developmental stage, the immigrant CD45+ hematopoietic cells colonize the epithelial-mesenchymal anlage of the BF, which results in LE follicle formation, and subsequently B-lymphocyte maturation (Nagy et al., 2004).

Sonic hedgehog (Shh) is expressed universally by the whole digestive endoderm, including the cloaca, but not the ectoderm (Roberts et al., 1995; Narita et al., 1998; Liu et al., 2007). Therefore, we examined the tail bud region and cloacal membrane for endoderm specific Shh protein and for expression of Shh and its Ptc1 receptor transcripts. At E5, the hindgut endoderm and the caudal wall of the cloaca are immunoreactive for Shh, unlike the ectoderm-derived anal sinus (Fig. 1C). Shh transcript is expressed in the hindgut and endodermal part of the cloacal epithelium (Fig. 1D), but not in bursal duct and epithelium (Fig. 1E). Ptc1 is present in the subepithelial mesenchyme of the distal hindgut but not in the bursa and bursal duct. (F) Ptc1 expression in the subepithelial mesenchyme of the distal hindgut but not in the bursa. hg, hindgut; nt, neural tube.

In order to study the contribution of tail ectoderm to the bursal epithelium, first we removed microsurgically the tail bud ectoderm of 16 HH chicken embryo. By E10, the anal invagination of control birds is clearly transformed to a proximal bursal duct and distal proctodeum (Fig. 2A) and the hematopoietic cells accumulate in the most proximal side of the bursa (Fig. 2B). Eight days after microsurgery in ectoderm-ablated embryos, there is no sign for bursal anlage in the ablated embryo. (B) In control embryos, CD45+ hematopoietic cells are scattered over the mesenchyme but they are accumulated at the most proximal part of the bursa (outlined). (D) In the ablated embryos, the CD45+ cells enter the tail bud mesenchyme, but they do not accumulate at a specific site.
Chicken-quail tail bud chimera and in vitro tissue recombination of chicken bursal mesenchyme with quail epithelium

In recombination experiments, the chick tail bud ectoderm was replaced by age-matched quail ectoderm, including the mesenchyme and neural tube (see Fig. S1 in the supplementary material). The chimeric embryos contain quail ectoderm, chick endoderm and hematopoietic cells, as well as mixed (quail and chick) mesoderm.

QCPN and cytokeratin immunostainings of the tail bud region indicate that the skin epithelium and the anal invagination with the primordium of the BF and part of the dorsal mesenchyme are derived from the transplanted quail ectoderm (Fig. 3A,B). Double staining of cytokeratin and CD45 indicates that chick hematopoietic cells enter the quail epithelium and initiate bud formation (Fig. 3C). These buds represent true tissue chimerism: the chick CD45+ cells colonize the quail-derived epithelium (Fig. 3C,D). The LE tissue of buds was further analyzed for differentiation of hematopoietic cells by using markers for B lymphocyte (Bu-1), immunoglobulin (IgM), chicken dendritic cell (74.3), MHC class II and granulocyte (Grl-1) (Fig. 3E,F,G,H,I). These chick-specific markers confirmed the formation of functional bursal follicles in these chimeras.

The next series of chimeric experiments were carried out to obtain information about the signaling role of the endoderm. In a pilot experiment, bursal follicle formation was tested by combining E8 chicken bursal mesenchyme with E8 quail bursal epithelium (see Fig. S2A,B,C in the supplementary material). The recombinant tissue (‘artificial’ bursal epithelial-mesenchymal anlage) was implanted into E3 chicken coelom for hematopoietic cell colonization and follicle formation. After 14 days of incubation, QCPN and 8F3 mAb stainings clearly indicate that the epithelium and mesenchyme including the hematopoietic cells of the BF are quail and chicken derived, respectively (see Fig. S2D,E,F in the supplementary material). These experiments provided evidence that chimeras are able to form follicles and LE tissue. Furthermore, the cells of the follicles express chick-specific antigens, such as Bu-1b, 74.3 and MHC class II (see Fig. S2G,H,I in the supplementary material).

Fig. 3. Chick-quail tail bud chimera. (A) Inset shows the tail bud chimeric embryo at day 14. The presence of pigmented and nonpigmented feathers covers the quail and chick derivatives, respectively. Sagittal section (white line) made from the chimeric tail bud is shows the QCPN+ quail cells (red) forming the surface epithelium, the bursal epithelial anlage and the mesenchyme under the skin. (B) Cytokeratin staining confirms the epithelium (blue). (C) Chicken CD45+ hematopoietic cells (green) are scattered over the mesenchyme and enter the epithelium (blue). (D) Another combination of immunostaining confirms that the quai epithelial bud (red, QCPN) is colonized by chicken hematopoietic cells (green, CD45). (E-I) The developing follicle contains phenotypically functional, Bu1b+AgM-producing B lymphocytes (E,F); (G) bursal secretory dendritic cells (74.3); and (H) MHC class II+ cells. However, granulocytes (Grl-1) appear only in the mesenchyme (I). Epithelial bud is outlined.

Fig. 4. Tissue recombination of chicken bursal mesenchyme with different quail epithelium. In vitro recombination of chicken bursal mesenchyme with (A) quail tail bud endoderm, (B) quail tail bud ectoderm, (C) quail trunk ectoderm and (D) hindgut endoderm. The recombinant tissue was implanted in the chick embryo coelomic cavity for hematopoietic cell colonization. QCPN shows that bursal epithelium is of quail origin, and the 8F3 and CD45 staining prove that the mesenchyme and hematopoietic cells are chick derived. Bu-1 immunostaining indicates the B lymphocytes. (B) Chicken mesenchyme is capable of follicle formation only with quail tail bud ectoderm.
In the next series of experiments, chick bursal mesenchyme was combined with either quail ectoderm or endoderm to determine which epithelial layer supports follicle formation (Fig. 4). In every tissue recombination, the ‘artificial’ bursal anlagen were capable of receiving CD45+ hematopoietic cells. Bu-1-positive B lymphocytes were present in all varieties of tissue recombinations, indicating that early B cell differentiation can take place without bursal micro-environment. However, follicle formation happened only if the chick bursal mesenchyme was recombined with quail tail bud ectoderm (Fig. 4B). In these chimeric experiments, the transplanted ectoderm was removed well before the formation of the anal plate; thus, endodermal instruction to ectodermal follicle formation is unlikely. Our findings obtained from the chimeric recombination experiments are reminiscent of those of Gordon et al. (Gordon et al., 2004) in stating that the thymic epithelial anlage does not require ectodermal signaling as the third branchial pouch can form functionally active thymic tissue without ectoderm. Similarly, tail bud ectoderm can form bursal follicles without endodermal contribution.

In birds, antigen-specific IgG production requires bursal LE tissue micro-environment. In the majority of mammals, B-cell maturation takes place in the bone marrow (Abdou and Abdou, 1972) or in the Peyer’s patches of ruminants (Reynaud et al., 1991), which are so-called bursa equivalent organs. Bone marrow histologically is not comparable with the LE tissue of BF. It seems likely that the B-lymphocyte maturation is not connected to a histologically determined structure, unlike T cells, which exclusively develop in the thymic LE tissue. If the bursa-equivalent organs of mammals are histologically not comparable with the LE tissue of BF, but the function of the immune system of birds and mammals is the same, it may be assumed that a `bursa function equivalent stromal cell' exists, which establishes the micro-environment for B cell differentiation. In jawed vertebrates, the dichotomous nature of lymphocytes (T and B cells are responsible for cellular and humoral immunity of adaptive immune response, respectively) is well established. While T cells develop exclusively in the endodermal-derived thymus, B lymphopoiesis occurs in different anatomical locations of vertebrate classes, i.e. pronephros, liver, spleen and gut-associated lymphoid tissue. In birds, the BF is a unique evolutionary invention for B-cell maturation. Thus, T- and B-lymphocyte maturation is orchestrated by endodermal and ectodermal-derived micro-environments, respectively.

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Competing interests statement
The authors declare no competing financial interests.

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